Published in final edited form as:
Eur J Immunol. 2012 December ; 42(12): 3136–3140. doi:10.1002/eji.201243061.

FcγRI (CD64): an identity card for intestinal macrophages

Jaime De Calisto, **Eduardo J. Villablanca**, and **J. Rodrigo Mora***
Gastrointestinal Unit, Massachusetts General Hospital, Harvard Medical School, Boston, MA 02114

Abstract

Macrophages (MF) are becoming increasingly recognized as key cellular players in intestinal immune homeostasis. However, differentiating MF from dendritic cells (DC) is often difficult, and finding a specific phenotypic signature for intestinal MF identification has remained elusive. In this issue of *European Journal of Immunology* Tamoutounour *et al.* identify CD64 as a specific MF marker capable of discriminating DC from MF in the murine small and large bowel, and under steady state and inflammatory conditions. The authors also propose a sequential 'monocyte (Mo)-waterfall' model for intestinal MF differentiation, with implications for immune tolerance and inflammation at the gut mucosal interface.

Mononuclear phagocytes, including MF and DC, are key components of innate and adaptive immune responses. While DC subsets have been thoroughly characterized in different tissues and under a variety of conditions, MF subsets and functions are much less understood. This is in great part due to the lack of markers to reliably identify and isolate MF in different tissues, including the gut. There is also a lack of consensus on how to functionally define DC and MF. DC were initially defined as antigen presenting cells with the capacity to activate naïve T cells. However, this definition is not accurate, as there are situations in which efficient T cell priming can occur in the absence of DC [1, 2]. Moreover, immature DC do not efficiently activate naïve T cells and therefore they could not be included in this functional definition. Thus, DC subsets are currently identified using a combination of phenotypic, functional, and ontogenetic criteria [3].

What is a macrophage?

MF are classically considered terminally differentiated non-migratory phagocytic cells, which are not involved in priming naïve T cells. However, recent data indicate that, beyond their phagocytic capacity, MF play an active and critical role in maintaining immune tolerance. In fact, under normal conditions intestinal MF are non-inflammatory scavengers of bacteria, and they are involved in expanding T_{REG} in the lamina propria (LP) [3, 4]. On the other hand, in some settings, such as during IBD, MF can become pro-inflammatory cells [3, 4].

In the gut MF have been usually identified by their expression of MHC-II, CD11b, fractalkine receptor (CX3CR1), F4/80, and by their lack of DC markers CD11c and CD103 [4]. However, CD11c can also be expressed at low/intermediate levels in intestinal LP MF, and some investigators have denominated MHC-II$^+$CD11b$^+$CD11c^{int} cells as MF [5, 6], while others dubbed them as DC [7]. On the other hand, although CX3CR1 is preferentially expressed in intestinal LP MF, some CD11b$^+$ DC exhibit intermediate levels of this receptor

*Correspondence should be addressed to: J. Rodrigo Mora, M.D., Ph.D., Gastrointestinal Unit, Massachusetts General Hospital, 55 Fruit St, GRJ-815, Boston, MA 02114, USA, Office: +1 (617) 643-4366, Fax: +1 (617) 849-5771, j_rodrigo_mora@hms.harvard.edu.

Conflicts of interest: The authors declare no competing financial interests.

[5]. Thus, there is an urgency to find specific phenotypic signatures capable of reliably discriminating MF and DC in the gut mucosa under different conditions.

In this context, Tamoutounour *et al.* propose using the high-affinity IgG receptor gamma chain FcγRI (CD64) to unambiguously label intestinal MF [8]. The authors show that CD64 can clearly discriminate MF from DC, even when MF express CD11c^{int} (CD64^{+}) or when DC express CX3CR1^{int} (CD64^{neg}) (Table 1). Of note, while granulocytes also express CD64, they can be excluded by their lack of MHC-II, high SSC, and their expression of Ly6G. Importantly, the authors show that CD64 can be used as a reliable MF marker in both small and large bowel, and under steady state or inflammatory conditions. Moreover, they propose that CD64 could also be used to label intestinal MF in humans. However, as discussed below, the expression of this marker in human MF might be less consistent than in the murine system. Also, given the potential for CD64 modulation under some conditions (discussed below), future work should assess whether the expression of this marker is consistently stable and specific in MF under different inflammatory or infectious scenarios.

Mo-waterfall model for intestinal MF differentiation: steady state versus gut inflammation

By combining CD64 with other classical markers described in Table 1, Tamoutounour *et al.* propose a model in which blood Mo (Ly6C^{high}MHC-II^{neg}CD64^{neg}) enter to the intestinal LP using CCR2. In the LP they lose Ly6C and reciprocally acquire MHC-II and CD64 expression [8]. Given the appearance of this labeling in the FACS plots, they described this differentiation pattern as 'Mo-waterfall' (Fig. 1A). A similar differentiation pathway was observed in a study that was published in parallel [9]. Of note, using elegant experiments involving *in vivo* labeling as well as adoptive transfer of labeled monocytes, Tamoutounour *et al.* convincingly demonstrated that the Mo-waterfall model of MF differentiation occurs in the steady state and during gut inflammation.

Unexpectedly, the authors show that during intestinal inflammation there is a massive recruitment of Mo to MLN, where they differentiate into MF [8]. Interestingly, in contrast to DC migration from LP to MLN via lymphatics, Mo recruitment to MLN occurs directly from the blood and not from the intestinal LP. In fact, while LP DC arrival to MLN is abrogated in CCR7$^{-/-}$ mice, macrophage expansion in MLN during inflammation was not affected in these mice [8]. Moreover, CCR7 was not required for triggering intestinal inflammation, suggesting that MF (and not LP DC) are the key pathogenic cells, at least in the tested IBD model. These observations challenge the conventional wisdom that intestinal inflammation needs to be pathogenically associated with DC migration from LP to MLN. It will be interesting to determine whether Mo entry to MLN also relies on CCR2. If so, it is tempting to speculate that intestinal inflammation might induce CCR2-ligands in MLN (or in IEC/LP, which then reach the MLN via lymphatics), hence recruiting CCR2^{+} blood monocytes, which will give rise to MLN MF.

The Mo-waterfall model distinguishes four distinct stages in LP MF differentiation (P1 to P4) [8, 9]. Interestingly, Tamoutounour *et al.* show that under inflammatory conditions MF differentiation is blunted, reaching only the P2 stage in the LP and MLN (F4/80^{neg}CX3CR1^{neg}, Table 1, Fig. 1B). P2 MF express iNOS and produce pro-inflammatory cytokines (IFNγ, TNFα) [8, 9]. How intestinal inflammation interferes and skews MF differentiation in the gut remains to be determined. In this regard, lack of CX3CR1 expression in P2 MF might preclude the generation of fully tolerogenic IL-10-producing MF, promoting a pro-inflammatory phenotype. Supporting this view, mice lacking CX3CR1 exhibit impaired oral tolerance and increased susceptibility to intestinal inflammation [10, 11], an effect that was correlated with a lack of IL-10 expressing MF in

$CX3CR1^{-/-}$ mice [10]. $CX3CR1^{-/-}$ mice also show a marked decrease in the numbers of MF in the gut [11], suggesting that CX3CR1 might be involved in MF survival/turnover in the LP. However, another report did not find a decrease in total MF in $CX3CR1^{-/-}$ mice, although IL-10 production by MF was impaired in these mice [10].

Of note, although CX3CR1 is upregulated *in vivo* in the intestinal mucosa, this receptor is not induced upon *in vitro* MF differentiation, suggesting that local microenvironmental signals are needed to induce CX3CR1. However, although CX3CR1 is upregulated in gut LP MF, it can also happen in extra-intestinal tissues, such as the lungs [12], implying that the signals involved in modulating this receptor might not necessarily be gut-specific.

This newly proposed Mo-waterfall model for intestinal MF differentiation also implies that $CX3CR1^{neg}$ (P1/P2) and $CX3CR1^{+/high}$ (P3/P4) MF, as well as pro-inflammatory (P2) or tolerogenic (P4) MF, can all be positioned in a continuum spectrum of MF differentiation in the LP, rather than each MF type being originated from independent precursors [8, 9]. Thus, this new work not only describes a convenient and reliable marker for gut MF identification and isolation in the mouse, but it also provides a novel conceptual framework that potentially unifies previously published observations on intestinal DC and MF.

CD64 specificity, regulation, and therapeutic implications

CD64 expression and specificity

While Tamoutounour *et al.* clearly show that CD64 is a specific marker for intestinal MF in the mouse, it should be noted that CD64 might not faithfully discriminate MF from DC in extra-intestinal tissues. For example, a recent paper identified a migratory population of $Ly6C^{high}CD64^{+}$ DC in murine muscle (migratory monocyte-derived lymph node DC) [13]. While this $Ly6C^{high}CD64^{+}$ DC population depended on CCR2 for their initial location in muscle, in contrast to intestinal MF these cells can migrate to the draining lymph node in a CCR7-dependent manner upon inflammation [13].

Moreover, in humans CD64 might not be expressed in all MF subsets in the intestinal LP. In this regard, Tamoutounour *et al.* show that CD64 is expressed on $CD14^{+}$ MF in humanized mice and in biopsies from IBD patients. While the authors do not show whether $CD14^{neg}CD11c^{neg}$ MF express CD64, other reports indicate that resting $CD14^{neg}$ MF do not express CD64 and that this marker is preferentially expressed in inflammatory $CD14^{+}$ MF in human IBD [9, 14, 15]. Furthermore, one report did not show CD64 expression in human intestinal MF, and only mild CD64 expression in vaginal MF [16]. While the reason for this discrepancy is unclear, it might be related to differences in MF isolation techniques or to the markers/gates used in each study. Finally, CD64 can be readily detected in human blood monocytes [17, 18], suggesting that its expression in humans might be more ubiquitous, especially outside the gut. In summary, in contrast to the murine model, CD64 might not be a universal or specific marker for human intestinal MF.

CD64 induction and regulation

CD64 can be induced in MF by IFNγ [19], while IL-4 suppresses its expression in human and murine MF [18, 20, 21]. Given that Th1 and Th2 cytokines reciprocally modulate CD64 (*in vitro* and *in vivo*), it will be interesting to assess whether MF differentially express CD64 in the context of Th1- or Th2-associated IBD models, or in mouse strains with a Th2 bias (e.g., Balb/c). Of note, IL-10 also upregulates CD64 [22] and, at least *in vitro*, it can prevent CD64 downmodulation by Th2 cytokines [23]. Given the relative abundance of IL-10 in the intestinal compartment (even during inflammation [9]), this cytokine might sustain CD64 expression on MF, regardless of the Th1/Th2 environment.

Therapeutic aspects

In vivo targeting of CD64 has been experimentally used to boost peripheral antigen-specific antibody immune responses [24]. On the other hand, CD64 has been proposed as a therapeutic target for eliminating activated MF in some inflammatory conditions, such as arthritis [24, 25]. A downside is that CD64 can also be subverted by some pathogens for invading MF and cause severe systemic infection [26].

Regarding immunomodulation, it has been described that immune complexes promote CD64 crosslinking in MF, increasing IL-10 and shutting down IL-12 production *in vitro* and *in vivo* [27, 28]. While these effects of CD64 modulation might potentially be exploited to increase tolerogenicity in the gut mucosa in the context of IBD, it has been shown that CD64 might also have a negative impact on some antibody-mediated therapies, at least *in vitro* [29]. In particular, therapeutically used anti-TNFα antibodies (Infliximab and Adalimumab) can stimulate CD64 via their Fc portion, inducing the production of pro-inflammatory cytokines by human MF cell lines. Of note, this pro-inflammatory effect was not observed with Certolizumab (anti-TNFα Fab', lacking the Fc fragment) [29]. Thus, blocking CD64 might have a synergistic effect when using some of these anti-TNFα therapies in IBD. However, given the seemingly antagonistic effects of CD64 modulation in different settings, at this point it cannot be predicted the final outcome of harnessing CD64 in a therapeutic context.

Concluding remarks

Tamoutounour *et al.* demonstrate that CD64 can be used as a reliable marker to define an 'identity card' for intestinal MF in the mouse. Future work needs to determine whether CD64 is sufficiently stable and specific to label MF under different inflammatory settings. The usefulness of CD64 in humans appears to be restricted to $CD14^+$ inflammatory MF, but this interpretation needs to be supported by additional work in human biopsies from patients with and without active IBD.

In addition, the authors provide a sequential 'Mo-waterfall' model to define MF differentiation stages in the intestinal LP. This conceptually new framework allows positioning pro-inflammatory and tolerogenic MF in a continuum spectrum of gut MF differentiation that can be disrupted during inflammation. Finally, while CD64 has been targeted to boost or dampen peripheral immune responses in different experimental models, additional data is needed to predict the outcome of modulating this pathway in the intestinal mucosa.

Acknowledgments

EJV was supported by a postdoctoral fellowship from Crohn's & Colitis Foundation of America (CCFA). JRM was supported by grants from CCFA, PF Grant Harvard University Center for AIDS Research P30 AI060354 (HU-CFAR), and NIH New Director's Innovator Award DP2 2009A054301. JRM is indebted to Ingrid Ramos for constant support.

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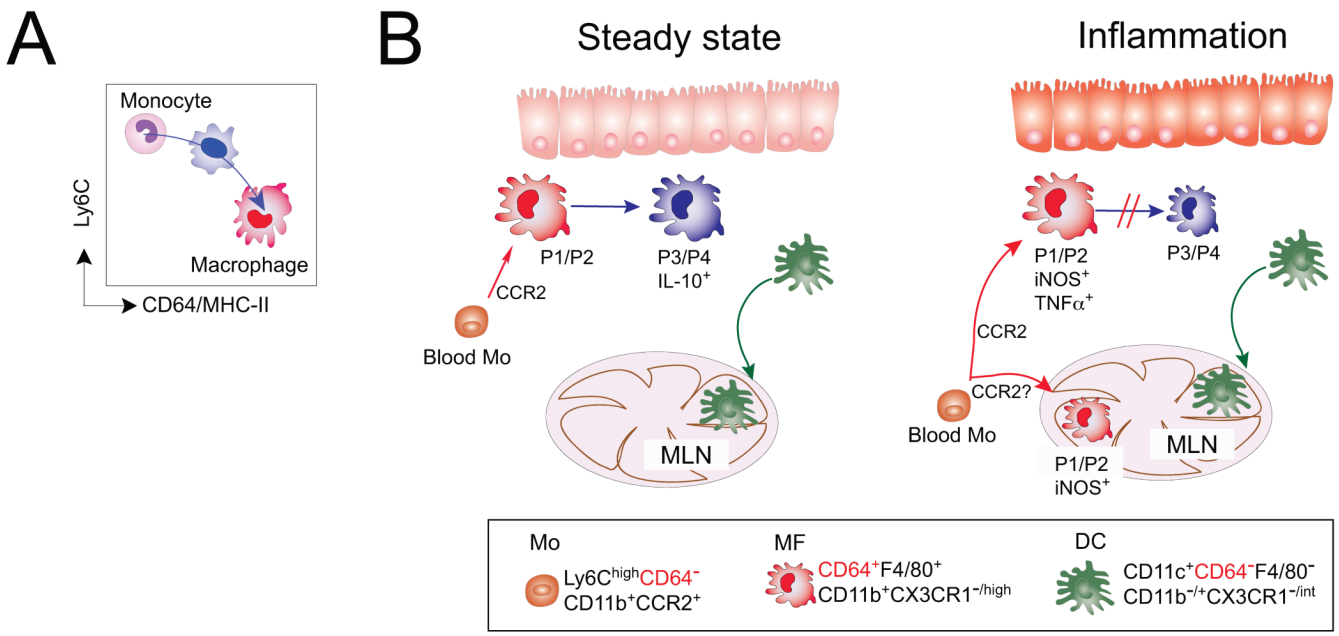

Figure 1. MF differentiation in the intestinal lamina propria

(**A**) 'Mo-waterfall' model (based on FACS plot pattern): Ly6C^{hi} blood monocytes (Mo) enter the intestinal lamina propria (LP) where they downregulate Ly6C and acquire the expression of MHC-II and CD64, becoming macrophages (MF). (**B**) Left: under steady state conditions, Mo enter to the LP in a CCR2-dependent manner. They first go through P1/P2 stages (CD64$^{low/+}$F4/80^{neg}CX3CR1$^{-/int}$) to finally become P3/P4 tolerogenic IL-10-producing MF (CD64^{+}F4/80^{+}CX3CR1^{high}). Right: during inflammation MF differentiation is blunted, reaching only the P2-MF stage and becoming pro-inflammatory iNOS^{+}TNFα^{+} MF. In this setting there is also massive P2-MF infiltration in mesenteric lymph nodes (MLN), which might also depend on CCR2.

Table 1

CD64 and its relationship with classical markers to identify intestinal DC and MF.

	LP DC	Blood Monocyte	MF differentiation stages in gut LP			
			P1 (immature)	P2	P3	P4 (differentiated)
CD64	−	+/−	+/−	++	++	++
CX3CR1	−/+	−	−	−/+	+	++
F4/80	−	−	−	−	+	+
CD11b	−/++	++	++	++	++	++
CD11c	+/++	−	−/+	−/+	−/+	−/+
MHC-II	++	−	−	+	++	++

(−) = negative; (+/−) = low; (−/+) = negative to intermediate; (+) = intermediate; (+/++) = intermediate to high; (++) = high